Enzyme, Bacterial Inoculant, and Formic Acid Effects on Silage Composition of Orchardgrass and Alfalfa[1,2]

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ABSTRACT

We evaluated the effects of cellulase (from *Trichoderma longibrachiatum*) application rates on neutral detergent fiber (NDF) concentration and fermentation products of orchardgrass (*Dactylis glomerata* L.) and alfalfa (*Medicago sativa* L.) silages harvested with decreasing dry matter (DM) digestibility. Additionally, the impacts of inoculant (*Lactobacillus plantarum* and *Pediococcus cerevisiae*), pectinase (from *Aspergillus niger*), or formic acid on silage composition were studied. Forages wilted to a DM content of about 320 g/kg were ensiled in laboratory silos for 60 d. Cellulase, combined with inoculant, was applied at 2, 10, and 20 ml/kg of herbage (at least 2500 IU/ml). Cellulase at 10 ml/kg was also applied alone or in combination with pectinase and inoculant or formic acid. The NDF concentration of orchardgrass silage decreased with increasing cellulase up to 20 ml/kg, at which NDF content was decreased by 30%. The NDF concentration of alfalfa silage decreased with increasing cellulase application up to 10 ml/kg, at which NDF content was decreased by 13%. Immature plants were more responsive to cellulase treatment than mature plants. Cellulase at 2 ml/kg combined with inoculant improved fermentation characteristics of the silages but generally, there was no effect on silage fermentation by higher cellulase applications, resulting in an accumulation of sugar. The improved fermentation of orchardgrass treated with cellulase and inoculant was mostly related to the effect of inoculant, whereas cellulase alone improved fermentation characteristics of alfalfa silage and this effect was enhanced by addition of inoculant. Decreased NDF and increased sugar concentrations did not improve the in vitro DM digestibility of cellulase-treated silages.
(**Key words:** silage, enzymes, cell wall, maturity)

Abbreviation key: ADL = acid detergent lignin, **IVDDM** = in vitro digestible dry matter.

INTRODUCTION

Cell-wall degrading enzymes, such as cellulases and hemicellulases, applied to herbage before ensiling can decrease cell-wall concentration of ensiled crops (9, 25, 28). Henderson et al. (8) and Nadeau (17) have shown greater enzymatic cell-wall hydrolysis in grasses than in legumes. Furthermore, enzymes have greater effects on cell- wall concentrations in immature than in mature plants (32). These differences are probably related to greater lignification in legumes than in grasses and to the increased lignification of cell walls as plants mature (3). Lignin in association with hemicellulose protects cellulose from enzymatic hydrolysis (7), and the rate of cellulose degradation is related to the amount of surface area accessible to cellulolytic enzymes (34). Pectins are embedded in the lignin-hemicellulose complex (7) and are present in greater amounts in legumes (200 to 300 g/kg of cell wall) than in grasses [<10 g/kg of cell wall (26)]. To further increase enzymatic degradation of cell walls during ensiling, pectinase in combination with cellulase and hemicellulase has been added to forages at the time of ensiling (11, 28, 30).

Enzymes are most beneficial on crops with low sugar concentrations, such as legumes and grasses with low DM concentrations (15). Sugars released during enzymatic cell-wall hydrolysis provide additional substrates for desirable lactic acid bacteria to produce lactic acid (15). High lactic acid production decreases pH to near 4.0 and restricts proteolytic activity (16). The addition

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of inoculants containing lactic acid bacteria can increase production of lactic acid and the rate of pH decline only when sufficient fermentable sugars are available (1, 10). Enzymes combined with an inoculant can improve fermentation and decrease proteolysis of silage (28, 30), whereas enzymes applied alone have had variable effects on silage fermentation (9, 25, 30).

In contrast to enzymes and inoculants that stimulate silage fermentation, formic acid restricts fermentation and decreases silage pH by direct acidification (15, 20, 33). Formic acid is commonly used in Scandinavia on crops with low DM and sugar concentrations. Under these conditions, it is especially important to decrease pH rapidly (<4.2) to prevent clostridial growth (14, 15).

Well fermented, highly digestible silages containing high concentrations of lactic acid, and low acetic acid, ammonia N, and cell wall concentrations are associated with higher intake and improved animal performance (2, 35). Others have reported effects of enzyme application rates, with or without bacterial inoculant, on silage composition (9, 12, 25, 30). The reported results have been inconsistent, however, and information about the effects of plant species and harvest dates on optimal enzyme application rates for increasing cell-wall degradation and improving fermentation of silage is lacking. Also, information about interactions among plant species, harvest date, and use of silage additives on the chemical composition of silage is limited.

The objective of this experiment was to determine the effects of cellulase application rates in combination with a bacterial inoculant on cell-wall degradation and fermentation products of orchardgrass (*Dactylis glomerata* L.) and alfalfa (*Medicago sativa* L.) silages harvested with increasing forage age and decreasing DM digestibility. Additionally, the effects of an inoculant, pectinase, and formic acid on silage composition were studied.

MATERIALS AND METHODS

Plant Material and Ensiling

Orchardgrass and alfalfa were grown in a split-split-split- plot design with four field replicates in a randomized complete block arrangement of treatments at the Agronomy and Agricultural Engineering Research Center of Iowa State University near Ames. Main plots representing plant species were 9 × 18 m, subplots representing growth cycles (spring and summer growths) were 4.5 × 18 m, and sub-subplots representing harvest dates were 4.5 × 6 m. The soil was a Webster (Typic Haplaquolls) fertilized with 230 kg of K and 50 kg of P/ha on July 1, 1992. Orchardgrass also was fertilized with 95 kg of N/ha on May 7, 1992, and with 65 kg of N/ha on July 1, 1992.

Table 1. Dry-matter, lignin, and CP concentrations of orchardgrass and alfalfa harvested at three consecutive dates averaged across forage treatments and growth cycles[1].

	Harvest[2]			
Species	1	2	3	$\bar{x}$
DM, g/kg				
Orchardgrass	304	318	354	325
Alfalfa	329	326	319	325
$\bar{x}$	317[b]	322[b]	336[a]	
Lignin, g/kg of DM				
Orchardgrass	39	36	41	39
Alfalfa	57	72	77	69***
$\bar{x}$	48[b]	54[a]	59[a]	
CP, g/kg of DM				
Orchardgrass	233	186	163	194
Alfalfa	250	216	210	225***
$\bar{x}$	241[a]	201[b]	187[c]	

[a,b,c]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]DM: species × harvest, $P < 0.001$, LSD (0.05) = 19. Lignin: species × harvest, $P < 0.001$, LSD (0.05) = 7. CP: species × harvest, $P < 0.001$, LSD (0.05) = 8.

[2]Harvest 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

***$P < 0.001$ for the main effect of species.

Forages were harvested with a sickle-bar mower on three dates at approximately 2-wk intervals during spring (May 22, June 5, and June19) and summer (July 16, August 4, and August 13) growth cycles in 1992. All plots were mowed on June 22 to allow for regrowth. The in vitro digestible DM (*IVDDM*) of orchardgrass at the three sequential harvest dates were 664 (two- to three-leaf stage), 657 (early heading), and 620 g/kg (late heading); the corresponding NDF concentrations were 556, 528, and 533 g/kg of DM during the spring growth cycle. In the summer growth cycle, the IVDDM of orchardgrass were 682, 666, and 601 g/kg and the NDF concentrations were 537, 562, and 627 g/kg of DM. Most of the orchardgrass plants did not produce reproductive heads during the summer growth cycle. The IVDDM of alfalfa at the three sequential harvest dates were 726, 694, and 646 g/kg during the spring growth cycle and 695, 663, and 603 g/kg during the summer growth cycle. The corresponding NDF concentrations of alfalfa were 327, 329, and 412 g/kg of DM during the spring growth cycle and 333, 411, and 442 g/kg of DM during the summer growth cycle. Alfalfa maturities at the three sequential harvest dates were early bud, early bloom, and late bloom for both growth cycles. For ensiling, forages were chopped to a 10-mm length with paper cutters and wilted to between 300 and 350 g of DM/kg of herbage (Table 1).

Additives used were cellulase, pectinase, bacterial inoculant, and formic acid. The liquid cellulase (Multifect CL, Genencor International, Inc., Rochester,

NY), which also had some hemicellulolytic activity, was derived from *Trichoderma longibrachiatum* and had a minimum carboxymethylcellulase activity of 2500 IU/ml (pH 4.8, 50°C) as stated by the manufacturer. The liquid pectinase (Cytolase PCL1, Genencor International, Inc., Rochester, NY) was derived from *Aspergillus niger* and had a minimum activity of 1300 apple pomace pectin viscosity units/ml (pH 3.8, 22°C) as stated by the manufacturer. Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) contained both *Lactobacillus plantarum* and *Pediococcus cerevisiae*. A water solution of the inoculant was applied at 10^5 cfu of lactic acid bacteria/g of herbage as recommended by the manufacturer.

The nine treatments were wilted herbage before ensiling; wilted control silage with no treatment; and silage made from wilted forage treated with inoculant plus cellulase at 2, 10, and 20 ml cellulase/kg of wilted herbage; cellulase (10 ml/kg) without inoculant; inoculant plus cellulase at 10 ml/kg combined plus pectinase at 0.3 and 3 μl pectinase/kg; and cellulase at 10 ml/kg combined with formic acid (88%) at 4 ml formic acid/kg wilted herbage. Cellulase application rates were chosen based on previous results with a dosage range from 0.3 to 2.4 ml of the liquid cellulase/kg of wilted herbage (18), whereas pectinase application rates were chosen based on recommendations from the manufacturer. Water was added to the control as well as to treated silage so that a total of 5% liquid was added to all wilted herbage weights. Additives were sprinkled separately over the wilted herbage, which was then mixed thoroughly. Immediately following treatment application, treated herbage (600 g) was packed in 946-ml glass jars (Qorpak, Pittsburgh, PA) used as laboratory silos. The silos (four silos/treatment) were sealed with lids lined with Teflon discs and equipped with fermentation traps containing water. Plant material was ensiled for 60 d at 20°C. When the silos were opened, the contents of each silo were mixed thoroughly before samples were taken. All samples then were kept frozen at –20°C until they were prepared for chemical analyses.

Chemical Analyses

One 100-g sample from wilted herbage and from each silo was freeze-dried and ground in a UDY cyclone mill (UDY Corporation, Fort Collins, CO) to pass a 1-mm screen for analysis of IVDDM, NDF, ADF, and acid detergent lignin (**ADL**). The DM concentration was determined by weighing samples before and after freeze-drying. The IVDDM analysis was based on the NC-64 direct acidification technique (13) and the buffer was flushed with CO_2 and reduced by the addition of sodium sulfide and cysteine hydrochloride. Resazurin, used as an oxidation-reduction indicator, changed from reddish pink to colorless when the buffer was reduced (6). Rumen fluid was collected from a cannulated steer fed a diet of orchardgrass and alfalfa hay.

Concentrations of NDF, ADF, and ADL were determined sequentially with α-amylase (Sigma Chemical Co, St. Louis, MO, No. A-6814) included in the NDF procedure (6, 31). Hemicellulose concentration was calculated as the difference between NDF and ADF concentrations, and cellulose concentration was calculated as the difference between ADF and the sum of ADL plus ADF insoluble ash concentrations. Because pectinase, inoculant, and formic acid had no effects on NDF concentration at the second harvest, analyses of ADF and ADL were not conducted on silages treated with cellulase alone or combined with pectinase and inoculant, or formic acid from the first and third harvests.

A second 100-g sample of wilted herbage and of each silage was diluted with 100 ml of deionized water, mixed in a Waring blender (model 1113, Waring Products Div., Winsted, CT) for 30 s, and squeezed through one layer of cheese cloth. Herbage and silage pH were determined with a glass electrode on the fresh plant extracts before the extracts were frozen for later analyses of the concentrations of reducing sugars, organic acids, and NH_3-N. Before analysis, plant extracts were centrifuged at 11,200 $\times$ *g* at 5°C for 10 min.

Concentrations of reducing sugars were determined by using the Nelson Somogyi procedure with glucose as a standard and absorbance determined at 660 nm with an ultrospec 4050 (LKB Biochrom Ltd., Cambridge, England) (21, 29). Reducing sugars were not analyzed on silages treated with cellulase and inoculant in combination with pectinase from the first and third harvests. Individual organic acids, including formic acid, were measured by gas chromatography (model 5890 GC, HP3396 Series II integrater, HP7673A auto sampler, Hewlett-Packard Co., Wilmington, DE) of butyl esters, which were prepared as described by Salanitro and Muirhead (23). Heptanoate was used as an internal standard, and the butyl esters were separated on a HP5 10 m $\times$ 530 μm glass column coated with 5% phenylmethyl silica (Hewlett-Packard Co.). A flame ionization detector was used and N was the carrier gas with a flow rate of 6.3 ml/min. Injection port temperature was 180°C, and the detector temperature was 270°C. The oven temperatures were regulated as follows: 50°C for 30 s, followed by an 8°C/min increase to 100°C, and a 30°C/min increase to a final temperature of 180°C. Organic acids were not analyzed on silages treated with pectinase from the first and third harvests because of no effect of the pectinase on pH and NH_3-N

concentration at all three harvests and on the organic acids at the second harvest.

Concentration of NH_3-N was determined according to the QuikChem Method No. 26-107-06-2-B with a salicylate-nitroprusside color reagent, by using an automated ion analyzer (QuikChem AE, Lachat Instruments, Milwaukee, WI). The CP concentration was determined on fresh samples by using the macro-Kjeldahl technique with a Tecator 1015 digestion block (Tecator AB, Höganäs, Sweden). Digested samples were analyzed for concentrations of total N according to the QuikChem method no. 15-107-06-2-B with a salicylate-nitroprusside color reagent by using an automated ion analyzer (QuikChem AE, Lachat Instruments).

Statistical Design

Data were analyzed via analysis of variance for a split-split-split-plot design with four replicates in a randomized complete block arrangement of treatments by using the general linear model procedure (PROC GLM) of SAS (24). Plant species (n = 2) were treated as the whole plot, growth cycle (n = 2) as the subplot, harvest date (n = 3) as the sub-subplot, and forage treatment (n = 9) as the sub-sub-subplot. The effect of plant species was tested by using the plant species × replicate interaction as the error term and the effects of growth cycle and its interaction with plant species were tested by using the plant species × growth cycle × replicate interaction as the error term. The effects of harvest date and its interactions with plant species and growth cycle were tested by using the interaction among plant species, growth cycle, harvest date, and replicate as the error term. Because the forage treatments from both growth cycles behaved similarly for the variables analyzed, data are presented as averages across growth cycles. Significant F tests at the 0.05, 0.01, and 0.001 levels of probability are reported. When a significant F value was detected, least significant difference at $P < 0.05$ was used to determine significant differences among means (5).

RESULTS AND DISCUSSION

Degradation of Cell Walls and Its Effect on DM Digestibility

Ensiling decreased NDF concentration in orchardgrass by 5% when averaged across harvests with, on average, 136% greater effect at the first harvest than at the following harvests (Table 2). The lower NDF concentration in control orchardgrass silage than in wilted herbage can be explained by acidic hydrolysis of hemicellulose, shown by a 17% lower hemicellulose concentration in control orchardgrass silage than in wilted herbage when averaged across harvests (Table 3). Selmer-Olsen et al. (25) reported similar decreases in NDF and hemicellulose concentrations during ensiling of perennial (*Lolium perenne* L.) and Italian ryegrass (*Lolium multiflorum* L.).

Because there were no differences in NDF degradation between cellulase alone and cellulase combined with inoculant, cell-wall degradation by the combination of cellulase and inoculant can be related to cellulase activity in this experiment (Table 2). Furthermore, research by Nadeau (17) reported no effect of inoculant on cell-wall concentration. Cellulase addition at 2 ml/kg decreased NDF and cellulose concentrations in both species (Tables 2 and 3). The NDF concentration in orchardgrass silage continued to decrease with increasing cellulase up to 20 ml/kg at the first and second harvests (Table 2). In the third harvest, NDF decreased significantly only up to 10 ml cellulase/kg of herbage. The response was smaller in alfalfa silage, with a significant decrease in NDF up to 10 ml of cellulase/kg at the first and third harvests, but only to 2 ml of cellulase/kg at the middle harvest (Table 2). Additionally, cellulose concentration in orchardgrass silage decreased significantly with increasing cellulase up to 20 ml/kg at the third harvest and to 10 ml of cellulase/kg at the first and second harvests (Table 3). In alfalfa, cellulose decreased significantly with increasing cellulase up to 10 ml/kg of herbage at the third harvest (Table 3). In agreement with our results, Jaakkola (9) and Selmer-Olsen et al. (25) reported decreased NDF and cellulose concentrations with increased application rates of a cellulase or a cellulase and hemicellulase mixture, respectively, applied to grass silage. In contrast to our results, Kung et al. (12) and Tengerdy et al. (30) found little or no effect of increased enzyme application on NDF and cellulose concentrations in alfalfa silage, when mixtures of cellulase, hemicellulase, and pectinase were used. These contrasting results may be caused by differences in DM concentrations of the silage and different application rates of the enzymes.

Averaged across harvests, cellulase at 20 ml/kg of herbage decreased NDF concentrations of orchardgrass and alfalfa silage by 30 and 14%, respectively (Table 2). The lower degradation of NDF by cellulase in alfalfa may be related to a 77% greater lignin concentration and a 33% lower initial NDF concentration in alfalfa than in orchardgrass (Tables 1 and 2). Similarly, cellulose degradation was twice as high in orchardgrass as in alfalfa silage (Table 3).

The NDF and cellulose degradation by cellulase (20 ml/kg) of orchardgrass silage decreased by 36 and 20%, respectively, from first to third harvest, and most of the decrease occurred between the second and third harvest (Tables 2 and 3). Because lignin concentration

Table 2. Neutral detergent fiber concentrations in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

	Harvest 1[2]		Harvest 2		Harvest 3		Species $\bar{x}$		Harvest $\bar{x}$			
Treatment[3]	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	1	2	3	Overall $\bar{x}$
	g/kg of DM											
WH	547	330	545	370	580	427	557	376	438	457	503	466[a]
Control	500	351	524	379	560	436	528	389	425	452	498	458[b]
IC2	392	325	429	349	511	409	444	361	359	389	460	402[c]
IC10	346	295	380	342	437	379	388	339	321	361	408	363[e]
IC20	321	284	359	338	431	385	371	336	302	349	408	353[f]
C10	332	308	379	343	436	391	382	347	320	361	414	365[e]
IC10P0.3	361	278	386	354	432	396	393	343	320	370	414	368[e]
IC10P3	338	311	376	347	447	390	387	349	325	361	418	368[e]
FAC10	359	324	388	348	435	400	394	358	341	368	418	376[d]
$\bar{x}$	388	312	419	352	474	401	427***	355	350[z]	385[y]	438[x]	

[a,b,c,d,e,f]Means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[x,y,z]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]Species × harvest, nonsignificant; species × treatment, $P < 0.001$, LSD (0.05) = 10; harvest × treatment, $P < 0.001$, LSD (0.05) = 13; species × harvest × treatment, $P < 0.001$, LSD (0.05) = 18.

[2]Harvests 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage; IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg; C10 = cellulase, 10 ml/kg; IC10P0.3, = inoculant + cellulase, 10 ml/kg + pectinase, 0.3 μl/kg; IC10P3 = inoculant + cellulase, 10 ml/kg + pectinase, 3 μl/kg; FAC10 = formic acid, 4 ml/kg + cellulase, 10 ml/kg. Cellulase: Multifect CL (Genencor International, Inc., Rochester, NY). Pectinase: Cytolase PCL1 (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

***$P < 0.001$ for the main effect of species.

Table 3. Cellulose and hemicellulose concentrations in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

Component and treatment[3]	Harvest 1[2]		Harvest 2		Harvest 3		Species $\bar{x}$		Harvest $\bar{x}$			
	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	1	2	3	Overall $\bar{x}$
	g/kg of DM											
Cellulose												
WH	257	201	255	208	274	257	262	222	229	231	265	242[a]
Control	248	199	275	212	291	263	271	225	224	243	277	248[a]
IC2	168	175	197	196	230	242	198	204	172	196	236	201[b]
IC10	130	158	169	194	208	204	169	185	144	181	206	177[c]
IC20	138	156	163	184	188	207	163	183	147	174	198	173[c]
$\bar{x}$	188	178	212	199	238	234	213*	204	183[z]	205[y]	236[x]	
Hemicellulose												
WH	235	63	253	97	247	88	245	83	149	175	168	164[a]
Control	194	81	206	88	211	87	204	85	137	147	149	144[b]
IC2	164	71	191	82	216	87	190	80	118	136	152	135[c]
IC10	155	64	172	74	183	78	170	72	110	123	130	121[d]
IC20	133	66	158	79	183	88	158	78	99	119	136	118[d]
$\bar{x}$	176	69	196	84	208	85	194***	80	123[z]	140[y]	147[x]	

[a,b,c]Cellulose; means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[a,b,c,d]Hemicellulose; means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[x,y,z]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]Cellulose: species × harvest, nonsignificant (NS); species × treatment, $P < 0.001$, LSD (0.05) = 11; harvest × treatment, $P < 0.05$, LSD (0.05) = 13; species × harvest × treatment, $P < 0.01$, LSD (0.05) = 18. Hemicellulose: species × harvest, $P < 0.05$, LSD (0.05) = 9; species × treatment, $P < 0.001$, LSD (0.05) = 9; harvest × treatment, $P < 0.01$, LSD (0.05) = 11; species × harvest × treatment, NS.

[2]Harvest 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage, IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg. Cellulase: Multifect CL (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

*$P < 0.05$ for the main effect of species for cellulose concentration.

***$P < 0.001$ for the main effect of species for hemicellulose concentration.

in orchardgrass was not affected by harvest date (Table 1), other factors, such as lignin structure and phenolic interactions with polysaccharides, may have limited cell-wall degradation in orchardgrass at later harvest dates (4). In alfalfa, degradation of NDF by cellulase (20 ml/kg) and cellulose decreased by 43 and 39%, respectively, between first and second harvests, which may be explained by a simultaneous 26% increase in lignin concentration in alfalfa (Tables 1, 2, and 3). Cellulose degradation by 20 ml/kg of cellulase in alfalfa silage increased by 61% between the second and third harvest (Table 3), without, however, any significant, simultaneous increase in NDF degradation (Table 2).

Averaged across harvests, hemicellulose concentration of orchardgrass silage decreased significantly with increasing cellulase up to 20 ml/kg, at which a 22% more degradation occurred than with the control (Table 3). Cellulase applied at 10 ml/kg decreased hemicellulose concentration in alfalfa by 15% (Table 3). Averaged across species, hemicellulose degradation by cellulase at 20 ml/kg decreased by 68% from the first to third harvest.

Beyond the effects of cellulase, pectinase and formic acid had no effects on NDF concentration, as indicated by no consistent differences between inoculant plus cellulase and inoculant plus cellulase combined with pectinase or cellulase alone and cellulase combined with formic acid (Table 2). Differences in cellulose and hemicellulose concentrations between these treatments were small and inconsistent. At the second harvest, cellulose and hemicellulose concentrations for cellulase (10 ml/kg) alone, inoculant plus cellulase (10 ml/kg) plus pectinase at 0.3 and 3 μl pectinase/kg of wilted herbage, and formic acid plus cellulase (10 ml/kg) were 174 and 166, 169 and 176, 174 and 163, and 168 and 177 g/kg of DM, respectively, for orchardgrass silage and 187 and 80, 192 and 89, 187 and 89, and 201 and 75 g/kg of DM, respectively, for alfalfa silage. Furthermore, other research by Nadeau (17) has shown no effects of formic acid on cell-wall concentration.

Despite extensive cell-wall degradation by cellulase, IVDDM was not increased in the cellulase-treated silages (Table 4). This is consistent with research by Van Vuuren et al. (32) and Nadeau (17); however, Russell (22) reported increased IVDDM in cellulase-treated corn (*Zea mays* L.) stover silage. The causes for contrasting results on IVDDM in the literature are unclear, but an effect of cellulase on digestibility after 48 h in vitro ruminal incubation is not expected (19). Nadeau et al. (19) reported greater total DM and NDF disappearances during early ruminal fermentation in situ in cellulase plus formic acid treated orchardgrass and alfalfa silages compared with control silage, but the differences between treatments became smaller as the fermentation proceeded up to 96 h of incubation.

There was no plant species × harvest × treatment interaction for IVDDM of the silages in the current study. Ensiling decreased IVDDM by 2% compared with wilted herbage when averaged across harvests and species (Table 4). Formic acid increased IVDDM slightly ($P < 0.001$) in cellulase-treated silage. Averaged across treatments, IVDDM of orchardgrass and alfalfa decreased by 9 and 11% from first to third harvest with a larger decrease between the last two harvests than between the first two harvests (orchardgrass: 660, 638, and 603 g/kg of DM; alfalfa: 697, 659, and 621 g/kg of DM for harvest 1, 2, and 3, respectively). Consequently, alfalfa had 6% greater IVDDM than orchardgrass at the first harvest, but alfalfa had only 3% greater IVDDM at the two following harvests.

Production of Reducing Sugars

Cellulolytic hydrolysis of the cell walls increased the concentration of reducing sugars in silages. Across species and harvests, each additional gram of NDF degradation resulted in an increase of 0.64 g of reducing sugars (Figure 1). The relationship was more evident in orchardgrass silage than in alfalfa silage because of the greater amount and broader range of NDF degraded in orchardgrass. Averaged across harvests, sugar concentrations in control alfalfa and orchardgrass silage increased by two and nine times, respectively, when cellulase was applied at 20 ml/kg (Table 5). This increased sugar concentration did not inhibit further cell-wall degradation during ensiling, as shown in orchardgrass silage (Table 2). Thus, decreased cell-wall degradation in alfalfa is probably related more to interactions among phenolics and polysaccharides in the cell walls than to product inhibition. Likewise, Jaakkola (9) found increased sugar concentration with increasing cellulase application to timothy (*Phleum pratense* L.) silage.

Across species, the average increase in sugar concentration caused by cellulase at the last two harvests was approximately 50% less than at the first harvest because of less NDF degradation at the later harvests (Tables 2 and 5). Because most of the sugars in wilted herbage were fermented to organic acids during ensiling, sugar concentrations in control orchardgrass and alfalfa silage were 72 and 62% lower than in wilted herbage of orchardgrass and alfalfa, when averaged across harvests (Tables 5, 6, 7, 8, and 9).

Much of the sugars present in the wilted herbage and sugars hydrolyzed by cellulase during ensiling was preserved by formic acid in the silage treated with formic acid plus cellulase. Consequently, orchardgrass and

Table 4. In vitro digestible DM in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

Treatment[3]	Species		Harvest[2]			Overall $\bar{x}$
	Orchardgrass	Alfalfa	1	2	3	
	g/kg					
WH	648	671	692	670	617	660[a]
Control	636	651	674	648	608	644[c]
IC2	630	660	677	649	609	645[bc]
IC10	633	660	677	648	614	646[bc]
IC20	631	658	679	647	608	645[bc]
C10	627	656	676	639	610	642[c]
IC10P0.3	627	657	675	636	615	642[c]
IC10P3	629	660	674	647	612	644[c]
FAC10	641	659	681	655	614	650[b]
$\bar{x}$	634	659***	678[x]	649[y]	612[z]	

[a,b,c]Means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[x,y,z]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]Species × treatment, nonsignificant; harvest × treatment, $P < 0.05$, OSD (0.05) = 10.

[2]Harvests 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage, IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg; C10 = cellulase, 10 ml/kg; IC10P0.3 = inoculant + cellulase, 10 ml/kg + pectinase, 0.3 μl/kg; IC10P3 = inoculant + cellulase, 10 ml/kg + pectinase, 3 μl/kg; FAC10 = formic acid, 4 ml/kg + cellulase, 10 ml/kg. Cellulase; Multifect CL (Genencor International, Inc., Rochester, NY). Pectinase; Cytolase PCL1 (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

***$P < 0.001$ for the main effect of species.

alfalfa silages treated with formic acid plus cellulase had 33 and 96% greater sugar concentrations, respectively, than orchardgrass and alfalfa silages treated with cellulase alone when averaged across harvests (Table 5). This is in agreement with results of Russell (22), who reported up to a 100% greater sugar concentration in silage treated with formic acid plus cellulase than in cellulase-treated corn stover silage. Across species, formic acid increased sugar concentration in cellulase-treated silage, on average, 124% more at the first harvest than at the two following harvests (Table 5). This increased sugar concentration in the silage treated with cellulase and formic acid may decrease the aerobic stability of the silage. However, if the silage is packed well to minimize airflow in the silo and to improve preservation of the forage, the risk for aerobic deterioration during feedout is decreased (16).

Because it had no effect on NDF, pectinase had little or no effect on sugar concentration at the second harvest (orchardgrass: 112 and 131 g/kg of DM; alfalfa: 36 and 37 g/kg of DM for inoculant plus cellulase (10 ml/kg) plus pectinase treatments at 0.3 and 3 μl pectinase/kg of wilted herbage, respectively). The concentration of pectinase added to the wilted herbage may not have been high enough to observe differences in cell-wall concentration, but, because we did not measure the concentration of pectin in the silages, it is difficult to identify the cause for lack of cell-wall degradation by pectinase.

Acidity and Acid Production

Ensiling resulted in a significant pH decrease, and the addition of cellulase alone decreased pH of control silage in both plant species (Table 6). Inoculant caused a further pH decline of cellulase-treated orchardgrass and alfalfa silages. Formic acid addition resulted in a higher pH of cellulase-treated silage, but the pH was still within the acceptable range (4.2 to 4.4) for a silage of good quality. Lactic acid concentrations did not increase and pH did not decrease with increased cellulase application, except for a slight trend in this direction for alfalfa silage at the second harvest (Tables 6 and 7). Consequently, increased cell-wall degradation caused by increased cellulase application resulted in accumulation of sugars that were not fermented (Table 5).

Cellulase at 2 ml/kg combined with inoculant increased lactic acid concentrations over control orchardgrass silage by 39 and 24% at the first and second harvest, respectively, and by 66% at the third harvest (Table 7). Alfalfa silage treated with cellulase at 2 ml/kg plus inoculant had 44 and 32% greater lactic acid concentrations than the control at the first and third

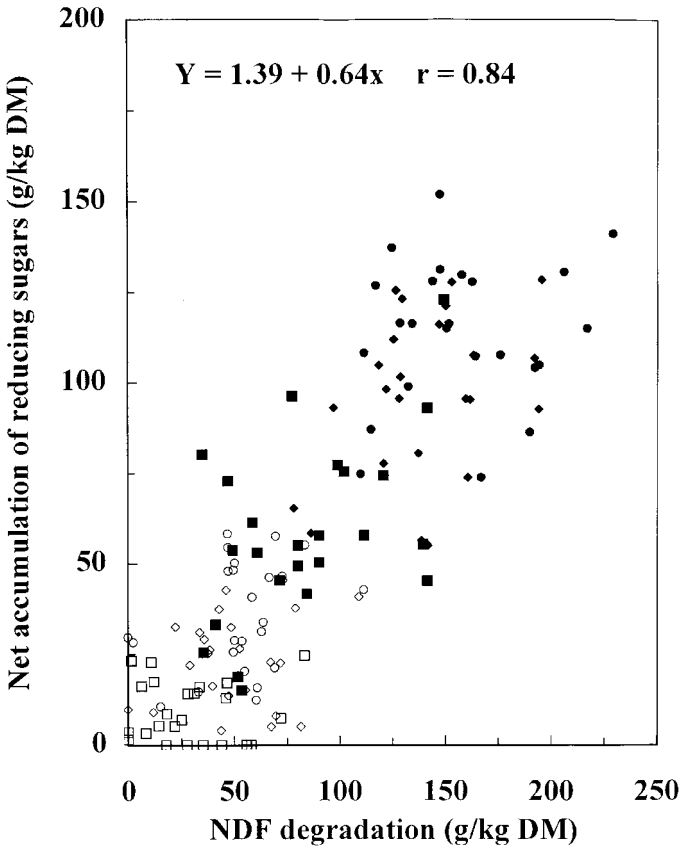

Figure 1. Net accumulation of reducing sugars as a function of NDF degradation (concentration of NDF in control silage – NDF in treated silages) in silages treated with cellulase (Multifect CL, Genencor International, Inc., Rochester, NY) at 2 (■, □), 10 (♦, ◇), and 20 (●, ○) ml/kg of wilted herbage of orchardgrass and alfalfa, respectively. Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied to cellulase-treated herbage at 10^5 cfu of lactic acid bacteria/g of wilted herbage. Data are values from each replicate.

harvest, respectively, and 79% greater lactic acid concentration at the second harvest.

Orchardgrass silage from the first harvest that was treated with cellulase at 10 ml/kg had a 30% greater lactic acid concentration than the control, with no additional effect of inoculant on lactic acid concentration (Table 7). However, inoculant increased lactic acid concentration in cellulase (10 ml/kg)-treated orchardgrass silage by 15 and 48% at the second and third harvest, respectively, with no effect of cellulase alone on lactic acid. In contrast to the orchardgrass results, cellulase applied alone to alfalfa silage increased lactic acid concentration significantly at all three harvests. Augmenting cellulase with inoculant produced additional lactic acid in alfalfa silage at the first two harvests. Increased lactic acid concentration by cellulase alone in alfalfa silage, but generally not in orchardgrass silage,

Table 5. Concentrations of reducing sugars in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

	Harvest 1[2]		Harvest 2		Harvest 3		Species $\bar{x}$		Harvest $\bar{x}$			
Treatment[3]	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	1	2	3	Overall $\bar{x}$
	g/kg of DM											
WH	40	44	53	48	45	49	46	47	42	51	47	47[d]
Control	7	13	16	25	17	17	13	18	10	20	17	16[e]
IC2	75	22	82	26	59	30	72	26	48	54	44	49[d]
IC10	107	41	122	38	99	46	109	42	74	80	73	76[c]
IC20	124	55	135	46	122	60	127	54	90	90	91	90[b]
C10	91	44	129	52	114	45	112	47	68	91	80	79[c]
FAC10	154	97	156	89	137	88	149	92	126	123	113	120[a]
$\bar{x}$	86	45	99	46	85	48	90***	46	65[y]	73[x]	66[y]	

[a,b,c,d,e]Means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[x,y]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]Species × harvest, nonsignificant (NS); species × treatment, $P < 0.001$, LSD (0.05) = 9; harvest × treatment, $P < 0.05$, LSD (0.05) = 10; species × harvest × treatment, NS.

[2]Harvests 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage, IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg; C10 = cellulase, 10 ml/kg; FAC10 = formic acid, 4 ml/kg + cellulase, 10 ml/kg. Cellulase; Multifect CL (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

***$P < 0.001$ for the main effect of species.

Table 6. The pH in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

	Harvest 1[2]		Harvest 2		Harvest 3		Species $\bar{x}$		Harvest $\bar{x}$			
Treatment[3]	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	1	2	3	Overall $\bar{x}$
WH	6.15	5.38	6.08	5.38	6.16	5.40	6.13	5.39	5.77	5.73	5.78	5.76[a]
Control	4.54	4.51	4.34	4.71	4.59	4.45	4.49	4.55	4.52	4.53	4.52	4.52[b]
IC2	4.01	4.09	4.02	4.19	4.05	4.02	4.03	4.10	4.05	4.10	4.04	4.06[e]
IC10	3.99	4.03	4.03	4.12	4.04	3.99	4.02	4.05	4.01	4.08	4.02	4.03[f]
IC20	3.98	4.03	4.01	4.05	4.00	3.97	4.00	4.01	4.00	4.03	3.99	4.01[f]
C10	4.07	4.17	4.17	4.31	4.42	4.08	4.22	4.19	4.12	4.24	4.25	4.20[d]
IC10P0.3	3.98	4.04	4.02	4.09	4.04	3.99	4.01	4.04	4.01	4.06	4.01	4.03[f]
IC10P3	3.97	4.03	4.00	4.09	4.04	3.98	4.01	4.04	4.00	4.05	4.01	4.02[f]
FAC10	4.25	4.38	4.28	4.40	4.42	4.31	4.31	4.36	4.31	4.34	4.36	4.34[c]
$\bar{x}$	4.33	4.30	4.33	4.37	4.42	4.24	4.36**	4.30	4.31	4.35	4.33	

[a,b,c,d,e,f]Means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[1]Species × harvest, $P < 0.001$, LSD (0.05) = 0.07; species × treatment, $P < 0.001$, LSD (0.05) = 0.05; harvest × treatment, $P < 0.01$, LSD (0.05) = 0.06; species × harvest × treatment, $P < 0.001$, LSD (0.05) = 0.08.

[2]Harvests 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage, IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg, C10 = cellulase, 10 ml/kg, IC10P0.3 = inoculant + cellulase, 10 ml/kg + pectinase, 0.3 μl/kg, IC10P3 = inoculant + cellulase, 10 ml/kg + pectinase, 3 μl/kg, FAC10 = formic acid, 4 ml/kg + cellulase, 10 ml/kg. Cellulase; Multifect CL (Genencor International, Inc., Rochester, NY). Pectinase; Cytolase PCL1 (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

**$P < 0.01$ for the main effect of species.

Table 7. Lactic acid concentrations in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

	Harvest 1[2]		Harvest 2		Harvest 3		Species $\bar{x}$		Harvest $\bar{x}$			
Treatment[3]	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	1	2	3	Overall $\bar{x}$
	g/kg of DM											
WH	0.0	1.3	1.2	0.2	0.1	0.0	0.4	0.5	0.7	0.7	0.1	0.5[e]
Control	62.6	49.9	63.2	30.9	44.3	47.5	56.7	42.8	56.2	47.0	45.9	49.7[c]
IC2	87.3	71.7	78.4	55.4	73.4	62.9	79.7	63.3	79.5	66.9	68.2	71.5[a]
IC10	82.3	74.9	73.3	63.5	72.4	64.0	76.0	67.5	78.6	68.4	68.2	71.8[a]
IC20	79.0	74.6	75.4	67.1	72.4	64.4	75.6	68.7	76.8	71.2	68.4	72.1[a]
C10	81.6	66.7	63.5	50.1	48.8	58.9	64.6	58.6	74.1	56.8	53.8	61.6[b]
FAC10	35.3	21.5	31.7	19.3	23.6	13.2	30.2	18.0	28.4	25.5	18.4	24.1[d]
$\bar{x}$	61.2	51.5	55.2	40.9	47.9	44.4	54.8**	45.6	56.3[x]	48.1[y]	46.1[y]	

[x,y]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]Species × harvest, $P < 0.05$, LSD (0.05) = 4.9; species × treatment, $P < 0.001$, LSD (0.05) = 3.7; harvest × treatment, $P < 0.001$, LSD (0.05) = 4.5; species × harvest × treatment, $P < 0.001$, LSD (0.05) = 6.4.

[2]Harvests 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage, IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg; C10 = cellulase, 10 ml/kg; FAC10 = formic acid, 4 ml/kg + cellulase 10 ml/kg. Cellulase; Multifect CL (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

**$P < 0.01$ for the main effect of species.

may be associated with a shortage of sugars for an optimal fermentation to occur in alfalfa silage treated with inoculant alone. Jaakkola (9) found increased lactic acid in cellulase- and cellulase and hemicellulase-treated grass silage, with or without addition of glucose oxidase, whereas Kung et al. (11, 12) reported no effect of cellulase or a cellulase and pectinase enzyme mixture on lactic acid concentrations in barley (*Hordeum vulgare* L.) and vetch (*Vicia villosa* Roth) silage, and alfalfa silage, respectively. Contrasting results can be explained by decreased NDF concentration in the enzyme-treated silage by Jaakkola (9) whereas Kung et al (11, 12) showed no effect of enzyme treatment on NDF concentrations of silages. There was, apparently, a shortage of sugars in the grass silage used by Jaakkola (9) for a successful fermentation to occur without addition of the enzyme.

Averaged across harvests, the addition of formic acid decreased lactic acid concentration in cellulase-treated (10 ml/kg) orchardgrass silage by 53%, with the greatest effect at the first harvest (Table 7). In alfalfa, formic acid plus cellulase-treated silage averaged 64% less lactic acid than cellulase-treated (10 ml/kg) silage at the first two harvests and 78% less at the third harvest. Pectinase had no effect on lactic acid concentration at the second harvest (orchardgrass: 76.6 and 76.0 g/kg of DM; alfalfa: 65.1 and 60.9 g/kg of DM for inoculant plus cellulase (10 ml/kg) plus pectinase at 0.3 and 3.0 μl pectinase/kg, respectively).

Averaged across treatments, lactic acid concentrations in orchardgrass were 19 and 35% greater than in alfalfa at the first and second harvest, respectively (Table 7). Lactic acid concentration in orchardgrass decreased by 22% from the first to the third harvest and the lactic acid concentration in alfalfa was on average 21% greater at the first harvest than at the second and third harvests (Table 7).

The cellulase (2 ml/kg) plus inoculant treatment decreased acetic acid concentrations over control orchardgrass silage by 38 and 45% at the first and second harvest, respectively (Table 8). The same treatment decreased acetic acid concentrations in alfalfa silage by 37 and 49% at the first and third harvest, respectively. An increase of cellulase application to 10 ml/kg was needed to cause a significant decrease in acetic acid concentration over control alfalfa silage at the second harvest. Cellulase applied at 20 ml/kg decreased acetic acid concentration over cellulase (2 ml/kg) plus inoculant treated alfalfa silage by 22 and 31% at the first and second harvest, respectively (Table 8). Likewise, Jaakkola (9) reported decreased acetic acid concentration with increased cellulase application rate to grass silage.

Table 8. Acetic acid concentrations in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

Treatment[3]	Harvest 1[2]		Harvest 2		Harvest 3		Species $\bar{x}$		Harvest $\bar{x}$			Overall $\bar{x}$
	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	1	2	3	
	g/kg of DM											
WH	1.2	1.4	1.1	1.3	1.1	0.9	1.2	1.2	1.3	1.2	1.0	1.2[d]
Control	14.6	17.4	11.4	17.3	9.0	16.7	11.7	17.1	16.0	14.4	12.8	14.4[a]
IC2	9.1	10.9	6.3	16.4	6.9	8.5	7.4	11.9	10.0	11.4	7.7	9.7[b]
IC10	8.5	9.0	7.9	14.5	7.6	8.1	8.0	10.5	8.8	11.2	7.8	9.3[b]
IC20	8.1	8.5	8.7	11.3	7.9	8.3	8.2	9.3	8.3	10.0	8.1	8.8[b]
C10	15.2	13.5	12.2	14.2	11.4	12.4	12.9	13.4	14.3	13.2	11.9	13.2[a]
FAC10	7.6	5.1	7.1	5.8	5.6	4.4	6.7	5.1	6.4	6.4	5.0	5.9[c]
$\bar{x}$	9.2	9.4	7.8	11.5	7.1	8.5	8.0	9.8*	9.3[x]	9.7[x]	7.8[y]	

[a,b,c,d]Means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[x,y]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]Species × harvest, $P < 0.01$, LSD (0.05) = 1.5; species × treatment, $P < 0.001$, LSD (0.05) = 1.4; harvest × treatment, $P < 0.05$, LSD (0.05) = 1.7; species × harvest × treatment, $P < 0.01$, LSD (0.05) = 2.4

[2]Harvests 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage, IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg; C10 = cellulase, 10 ml/kg; FAC10 = formic acid, 4 ml/kg + cellulase 10 ml/kg. Cellulase; Multifect CL (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

*$P < 0.05$ for the main effect of species.

Cellulase applied alone decreased acetic acid concentrations compared with control alfalfa silage by 22, 18, and 26% at the first, second, and third harvest, respectively, whereas the same treatment generally had no effect on acetic acid concentrations in orchardgrass silage (Table 8). Averaged across harvests, inoculant decreased acetic acid concentration in cellulase-treated (10 ml/kg) orchardgrass silage by 38%, and the effect was smaller at later harvest dates. Inoculant decreased acetic acid concentration in cellulase-treated (10 ml/kg) alfalfa silage by an average of 34% at the first and third harvest. In agreement with our results, Jones et al. (10) and Sharp et al. (27) found decreased acetic acid concentration in inoculated alfalfa and ryegrass silage, respectively. Conversely, Kung et al. (12) reported increased acetic acid concentration in cellulase/pectinase-treated alfalfa silage.

Averaged across harvests, formic acid addition decreased acetic acid concentrations in cellulase-treated (10 ml/kg) orchardgrass and alfalfa silage by 48 and 62%, respectively (Table 3). Pectinase had no effect on acetic acid concentration at the second harvest (orchardgrass: 8.0 and 7.7 g/kg of DM; alfalfa: 12.5 and 12.4 g/kg of DM for inoculant plus cellulase (10 ml/kg) plus pectinase at 0.3 and 3.0 μl of pectinase/kg, respectively). Averaged across treatments and harvests, alfalfa had a 22% greater acetic acid concentration than orchardgrass (Table 8).

Most of the added formic acid before ensiling (~13 g formic acid/kg of DM) was recovered as formic acid in the formic acid plus cellulase-treated silages (orchardgrass: 2.9 and 13.7 g/kg of DM; alfalfa: 1.1 and 12.0 g/kg of DM for cellulase alone and combined with formic acid, respectively).

The cellulase (2 ml/kg) plus inoculant treatment increased total acid concentrations in control orchardgrass silage by 22 and 10% at the first and second harvest, respectively, and by 42% at the third harvest (Table 9). In alfalfa, the same treatment increased total acid concentration over control silage by 34% at the second harvest and by 17 and 15% at the first and third harvest, respectively. These increases in total acid concentration follow the same trend as the increases in lactic acid production in orchardgrass and alfalfa silages treated with cellulase at 2 ml/kg plus inoculant (Tables 7 and 9). Similarly to pH and lactic acid, an increase in the cellulase rate generally did not increase total acid concentration (Tables 6, 7, and 9). Thus, cellulase applied at 2 ml/kg supplied enough sugar to improve silage fermentation (Table 5). When cellulase was applied alone to orchardgrass silage, total acid concentrations increased by 23 and 15% over control silage at the first and third harvest, respectively (Table 9). Also, alfalfa silage treated with cellulase alone had 14, 24,

Table 9. Total acid concentrations in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

	Harvest 1[2]		Harvest 2		Harvest 3		Species $\bar{x}$		Harvest $\bar{x}$			
Treatment[3]	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	1	2	3	Overall $\bar{x}$
	g/kg of DM											
WH	11.9	23.9	11.6	23.8	12.2	17.8	11.9	21.8	17.9	17.7	15.0	16.9[e]
Control	85.3	85.1	84.4	66.5	62.1	74.4	77.3	75.4	85.2	75.5	68.3	76.3[c]
IC2	104.4	99.6	92.5	89.0	88.0	85.3	95.0	91.3	102.0	90.8	86.7	93.1[a]
IC10	98.9	100.9	90.9	95.3	88.2	88.0	92.7	94.8	99.9	93.1	88.1	93.7[a]
IC20	94.9	100.3	93.7	96.5	89.6	87.6	92.7	94.8	97.6	95.1	88.6	93.7[a]
C10	104.8	97.4	87.6	82.5	71.5	85.7	88.0	88.5	101.1	85.0	78.6	88.2[b]
FAC10	60.5	52.6	57.4	49.2	46.6	42.8	54.9	48.2	56.6	53.3	44.7	51.5[d]
$\bar{x}$	80.1	80.0	74.0	71.8	65.5	68.8	73.2	73.5	80.0[x]	72.9[y]	67.1[z]	

[a,b,c,d,e]Means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[x,y,z]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]Species × harvest, nonsignificant; species × treatment, $P < 0.001$, LSD (0.05) = 3.9; harvest × treatment, $P < 0.001$, LSD (0.05) = 4.7; species × harvest × treatment, $P < 0.001$, LSD (0.05) = 6.7.

[2]Harvests 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage, IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg; C10 = cellulase, 10 ml/kg; FAC10 = formic acid, 4 ml/kg + cellulase, 10 ml/kg. Cellulase; Multifect CL (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

and 15% greater total acid concentrations than control silage at the first, second, and third harvest, respectively. Similarly, Jaakkola (9) reported increased total acid concentration in enzyme-treated grass silage.

Inoculant increased total acid concentration in cellulase-treated alfalfa silage at the second harvest (16%), and in cellulase-treated orchardgrass silage at the third harvest (23%; Table 9). Formic acid restricted total fermentation and decreased total acid concentration in cellulase-treated (10 ml/kg) orchardgrass silage by 42% at the first harvest and by 34 and 35% at the last two harvests. When formic acid was added to cellulase-treated (10 ml/kg) alfalfa silage, total acid concentrations decreased by 46, 40, and 50% at the first, second, and third harvest, respectively. Restricted fermentation products in grass and alfalfa silage by formic acid has been reported by others (9, 20, 33). Pectinase had no effect on total acid concentration at the second harvest (orchardgrass: 94.4 and 92.4 g/kg of DM; alfalfa: 96.3 and 91.5 g/kg of DM for inoculant plus cellulase (10 ml/kg) plus pectinase treatment at 0.3 and 3.0 μl pectinase/kg, respectively). Lack of response by pectinase on the acid concentrations is supported by our finding that pectinase had no effect on cell-wall concentrations of the silages. Averaged across treatments and plant species, total acid concentration decreased 16% during the course of harvests.

Degradation of Protein

Averaged across treatments, CP concentration decreased by 30 and 16% from first to third harvest in orchardgrass and alfalfa, respectively, and most of the decrease occurred between the first two harvests (Table 1). There were no or only slight differences in CP concentrations among the forage treatments of orchardgrass and alfalfa (data not shown).

Ammonia N as a portion of total N increased two to four times during ensiling, with a greater increase in alfalfa than in orchardgrass silage at the last two harvests (Table 10). Averaged across harvests, application of cellulase at 2 ml/kg combined with inoculant decreased NH_3-N concentration over control orchardgrass silage by 26%, with smaller effects at later harvest dates. In alfalfa, the same treatment decreased NH_3-N concentration by 16% compared with the control, with greater effects at later harvest dates.

Because there was generally no effect of increasing cellulase on pH and lactic acid, NH_3-N concentration was not affected by increased cellulase application (Tables 6, 7, and 10). Averaged across harvests, inoculant decreased NH_3-N concentration by 22% in orchardgrass silage treated with cellulase alone at 10 ml/kg, with only small differences among harvests (Table 10). Orchardgrass silage treated with cellulase alone at 10 ml/kg decreased NH_3-N concentration compared with the control (15%) only for the first harvest, whereas the same treatment decreased NH_3-N concentration of control alfalfa silage at the last two harvests (9 and 14%). Additionally, inoculant decreased NH_3-N concentration in alfalfa silage treated with cellulase at 10 ml/kg by 10 and 9% at the first and second harvest, respectively. The greater effect of inoculant than of cellulase on NH_3-N concentration in orchardgrass silage is consistent with our earlier work (18). The decreased NH_3-N concentration in inoculated silages was caused by a simultaneous increase in lactic acid production, which decreased pH and, therefore, proteolysis (Tables 6, 7, and 10). Also, the cellulase application provided more sugars for the lactic acid bacteria to produce lactic acid. In agreement with our results, Kung et al. (11) found decreased proteolytic activity when Biomate inoculant was applied to barley and hairy vetch silage at three stages of maturity, whereas Bolsen et al. (1) reported no effect of Biomate on NH_3-N concentration in alfalfa silage, when averaged over three maturities. Lack of response on NH_3-N concentration in the experiment by Bolsen et al. (1) is related to no effect of the inoculant on pH and lactic acid concentration of the silage after 90 d of ensiling.

Averaged across harvests, formic acid decreased NH_3-N concentration in cellulase-treated orchardgrass and alfalfa silage by 18 and 26%, respectively, with only small differences among harvests (Table 10). Others have also found decreased proteolytic activity in formic acid treated grass and alfalfa silage (9, 25, 33).

Averaged across treatments, orchardgrass had 11% lower NH_3-N concentration at the third harvest than at the first two harvests (Table 10). Alfalfa averaged 21% greater NH_3-N concentration at the middle harvest than at the first and third harvest.

CONCLUSIONS

For a maximal decrease of NDF concentration, an application of at least 20 ml cellulase/kg of herbage to orchardgrass and no more than 10 ml cellulase/kg to alfalfa can be used, when the cellulase has a minimum carboxymethylcellulase activity of 2500 IU/ml. Immature plants from the first and second harvests were more responsive than the more mature plants from the third harvest to increasing cellulase because they had cell walls that were more degradable. However, there were, generally, no effects of increasing cellulase on the fermentation products. Thus, cellulase applied at 2 ml/kg of herbage is sufficient to use to improve silage fermentation. Addition of inoculant to cellulase- treated orchardgrass silage enhanced homolactic fermentation

Table 10. Ammonia-nitrogen concentrations in wilted herbage and silage of orchardgrass and alfalfa harvested at three consecutive dates averaged across growth cycles.[1]

	Harvest 1[2]		Harvest 2		Harvest 3		Species $\bar{x}$		Harvest $\bar{x}$			
Treatment[3]	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	Orchardgrass	Alfalfa	1	2	3	Overall $\bar{x}$
	g/kg of total N											
WH	22.4	20.7	22.5	19.0	23.5	18.6	22.8	19.4	21.5	20.7	21.1	21.1[e]
Control	92.6	80.8	80.1	103.5	67.4	83.7	80.0	89.3	86.7	91.8	75.5	84.7[a]
IC2	64.9	72.4	59.7	86.1	53.5	66.6	59.4	75.0	68.7	72.9	60.0	67.2[c]
IC10	60.1	69.1	66.4	85.2	57.8	66.8	61.4	73.7	64.6	75.8	62.3	67.6[c]
IC20	61.6	71.9	63.4	79.9	58.7	66.9	61.2	72.9	66.8	71.7	62.8	67.1[c]
C10	78.7	76.4	85.8	93.7	71.0	72.4	78.5	80.8	77.6	89.8	71.7	79.7[b]
IC10P0.3	63.3	68.4	63.4	81.2	58.2	67.5	61.6	72.4	65.9	72.3	62.8	67.0[c]
IC10P3	62.4	69.9	64.0	82.0	57.8	65.4	61.4	72.4	66.2	73.0	61.6	66.9[c]
FAC10	64.5	56.1	69.2	67.2	60.5	55.2	64.7	59.5	60.3	68.2	57.8	62.1[d]
$\bar{x}$	63.4	65.1	63.8	77.5	56.5	62.6	61.2	68.4	64.2[y]	70.7[x]	59.5[z]	

[a,b,c,d,e]Means with different superscripts in the same column differ ($P < 0.05$) according to LSD test.

[x,y,z]Harvest means with different superscripts in the same row differ ($P < 0.05$) according to LSD test.

[1]Species × harvest, $P < 0.05$, LSD (0.05) = 5.9; species × treatment, $P < 0.001$, LSD (0.05) = 3.8; harvest × treatment, $P < 0.001$, LSD (0.05) = 4.7; species × harvest × treatment, $P < 0.001$, LSD (0.05) = 6.6.

[2]Harvests 1, 2, and 3 occurred on May 22, June 5, and June 19, respectively, for the spring growth cycle and on July 16, August 4, and August 13, respectively, for the summer growth cycle.

[3]WH = Wilted herbage, IC2 = inoculant + cellulase, 2 ml/kg; IC10 = inoculant + cellulase, 10 ml/kg; IC20 = inoculant + cellulase, 20 ml/kg; C10 = cellulase, 10 ml/kg; IC10P0.3 = inoculant + cellulase, 10 ml/kg + pectinase, 0.3 μl/kg; IC10P3 = inoculant + cellulase, 10 ml/kg + pectinase, 3 μl/kg; FAC10 = formic acid, 4 ml/kg + cellulase, 10 ml/kg. Cellulase; Multifect CL (Genencor International, Inc., Rochester, NY). Pectinase; Cytolase PCL1 (Genencor International, Inc., Rochester, NY). Bacterial inoculant (Biomate SI Forage Inoculant, Chr. Hansen's Laboratory, Inc., Milwaukee, WI) was applied at 10^5 cfu of lactic acid bacteria/g of wilted herbage.

and decreased proteolysis. As we have concluded in our earlier work, the cellulase and inoculant are most likely crop specific (18). Therefore, when biological silage additives are used, the product that is most suitable for the crop being ensiled should be chosen. Addition of formic acid restricted fermentation of sugars released from cell-wall degradation and of sugars already present in the herbage and its effects on the fermentation products were greater in alfalfa than in orchardgrass silage. Lack of response on IVDDM to decreased NDF and increased sugar concentrations was probably related to the less digestible cell walls remaining in the silage after hydrolysis by cellulase during ensiling (19). As all treated silages were preserved well regardless of harvest date, it is reasonable to harvest the forages at a date when DM digestibility of the forages is high enough to ensure sufficient forage intake by the ruminant animal.

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REFERENCES

1 Bolsen, K. K., C. Lin, B. E. Brent, A. M. Feyerherm, J. E. Urban, and W. R. Aimutis. 1992. Effect of silage additives on the microbial succession and fermentation process of alfalfa and corn silages. J. Dairy Sci. 75:3066–3083.

2 Buxton, D. R., and D. R. Mertens. 1995. Quality-related characteristics of forages. Pages 83–96 *in* Forages: The Science of Grassland Agriculture. Vol. 2. 5th ed. R. F. Barnes, D. A. Miller, and C. J. Nelson, ed. Iowa State Univ. Press, Ames, IA.

3 Buxton, D. R., and J. R. Russell. 1988. Lignin constituents and cell-wall digestibility of grass and legume stems. Crop Sci. 28:553–558.

4 Chesson, A. 1993. Mechanistic models in forage cell wall degradation. Pages 347–376 *in* Forage Cell Wall Structure and Digestibility. H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. Am. Soc. Agron., Crop Sci. Soc. Am., Soil Sci. Soc. Am., Madison, WI.

5 Cochran, W. G., and G. M. Cox. 1957. Experimental Designs. 2nd ed. John Wiley & Sons, Inc, New York, NY.

6 Goering, H. K., and P. J. Van Soest. 1970. Forage Fiber Analyses (Apparatus, Reagents, Procedures, and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC.

7 Hatfield, R. D. 1993. Cell wall polysaccharide interactions and degradability. Pages 285–313 *in* Forage Cell Wall Structure and Digestibility. H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. Am. Soc. Agron., Crop Sci. Soc. Am., Soil Sci. Soc. Am., Madison, WI.

8 Henderson, A. R., P. McDonald, and D. Anderson. 1982. The effect of a cellulase preparation derived from *Trichoderma viride* on the chemical changes during the ensilage of grass, lucerne and clover. J. Sci. Food Agric. 33:16–20.

9 Jaakkola, S. 1990. The effect of cell wall degrading enzymes on the preservation of grass and on the silage intake and digestibility in sheep. J. Agric. Sci. Finland 62:51–62.

10 Jones, B. A., L. D. Satter, and R. E. Muck. 1992. Influence of bacterial inoculant and substrate addition to lucerne ensiled at different dry matter contents. Grass Forage Sci. 47:19–27.

11 Kung, L. Jr., B. R. Carmean, and R. S. Tung. 1990. Microbial inoculation or cellulase enzyme treatment of barley and vetch silage harvested at three maturities J. Dairy Sci. 73:1304–1311.

12 Kung, L. Jr., R. S. Tung, K. G. Maciorowski, K. Buffum, K. Knutsen, and W. R. Aimutis. 1991. Effects of plant cell-wall- degrading enzymes and lactic acid bacteria on silage fermentation and composition. J. Dairy Sci. 74:4284–4296.

13 Marten, G. C., and R. F. Barnes. 1980. Prediction of energy digestibility of forages with in vitro rumen fermentation and fungal enzyme systems. Pages 61–71 *in* Proc. Int. Workshop on Standardization Anal. Methodol. Feeds. Ottawa, ON, Canada. W. J. Pigden, C. C. Balch, and M. Graham, ed. Unipub, New York, NY.

14 McDonald, P., A. R. Henderson, and S. J. E. Heron. 1991. The Biochemistry of Silage. 2nd ed. Chalcombe Publ., Marlow, Bucks, England.

15 Muck, R .E. and L. Kung, Jr. 1997. Effects of silage additives on ensiling. Pages 187–199 *in* Proc. Natl. Silage Prod. Conf., Hershey, PA. NRAES-99. Northeast Reg. Agric. Ext. Serv., Ithaca, NY.

16 Muck, R. E., and R. E. Pitt. 1993. Ensiling and its effect on crop quality. Pages 57–66 *in* Proc. Natl. Silage Prod. Conf., Syracuse, NY. NRAES-67. Northeast Reg. Agric. Ext. Serv., Ithaca, NY.

17 Nadeau, E.M.G. 1995. Enzyme, Inoculant, and Formic Acid Effects on Silage Quality of Orchardgrass and Alfalfa. Ph.D. Diss., Iowa State Univ., Ames (Diss. Abstr. 96-10975).

18 Nadeau, E.M.G., and D. R. Buxton. 1997. Cellulase and bacterial inoculant effects on cocksfoot and lucerne ensiled at high dry matter levels. J. Sci. Food Agric. 73:369–376.

19 Nadeau, E.M.G., D. R. Buxton, E. Lindgren, and P. Lingvall. 1996. Kinetics of cell-wall digestion of orchardgrass and alfalfa silages treated with cellulase and formic acid. J. Dairy Sci. 79:2207–2216.

20 Nagel, S. A., and G. A. Broderick. 1992. Effect of formic acid or formaldehyde treatment of alfalfa silage on nutrient utilization by dairy cows. J. Dairy Sci. 75:140–154.

21 Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375–380.

22 Russell, J. R. 1985. Effect of cellulase treatment on the nutritive value of corn stover. Pages 111–115 *in* Beef Cattle Research Report. AS-553. Coop. Ext. Serv., Iowa State University, Ames.

23 Salanitro, J. P., and P. A. Muirhead. 1975. Quantitative method for the gas chromatographic analysis of short-chain monocarboxylic and dicarboxylic acids in fermentation media. Appl. Microbiol. 29:374–381.

24 SAS User's Guide: Statistics, Version 5 Edition. 1985. SAS Inst., Inc., Cary, NC.

25 Selmer-Olsen, I., A. R. Henderson, S. Robertson, and R. McGinn. 1993. Cell wall degrading enzymes for silage. 1. The fermentation of enzyme-treated ryegrass in laboratory silos. Grass Forage Sci. 48:45–54.

26 Selvendran, R. R. 1983. The chemistry of plant cell walls. Pages 95–147 *in* Dietary Fibre. G. G. Birch and K. J. Parker ed., Appl. Science Publishers, New York, NY.

27 Sharp, R., P. G. Hooper, and D. G. Armstrong. 1994. The digestion of grass silages produced using inoculants of lactic acid bacteria. Grass Forage Sci. 49:42–53.

28 Sheperd, A. C., M. Maslanka, D. Quinn, and L. Kung, Jr. 1995. Additives containing bacteria and enzymes for alfalfa silage. J. Dairy Sci. 78:565–572.

29 Somogyi, M. 1945. A new reagent for the determination of sugars. J. Biol. Chem. 160:61–68.

30 Tengerdy, R. P., Z. G. Weinberg, G. Szakacs, M. Wu, J. C. Linden, L. L. Henk, and D. E. Johnson. 1991. Ensiling alfalfa with addi-

tives of lactic acid bacteria and enzymes. J. Sci. Food Agric. 55:215–228.
31 Van Soest, P. J., and J. B. Robertson. 1980. Systems of analysis for evaluating fibrous feeds. Pages 49–60 *in* Proc. Int. Workshop Standardization Anal. Methodol. Feeds. Ottawa, ON, Canada. W .J. Pigden, C. C. Balch, and M. Graham, ed. Unipub, New York, NY.
32 Van Vuuren, A. M., K. Bergsma, F. Frol-Kramer, and J.A.C. Van Beers. 1989. Effects of addition of cell wall degrading enzymes on the chemical composition and the in sacco degradation of grass silage. Grass Forage Sci. 44:223–230.
33 Waldo, D. R., J. E. Keys Jr., L. W. Smith, and C. H. Gordon. 1971. Effect of formic acid on recovery, intake, digestibility, and growth from unwilted silage. J. Dairy Sci. 54:77–84.
34 Weimer, P. J., J. M. Lopez-Guisa, and A. D. French. 1990. Effect of cellulose fine structure on kinetics of its digestion by mixed ruminal microorganisms in vitro. Appl. Environ. Microbiol. 56:2421–2429.
35 Wilkins, R. J., K. J. Hutchinson, R. F. Wilson, and C. E. Harris. 1971. The voluntary intake of silage by sheep. I. Interrelationships between silage composition and intake. J. Agric. Sci. (Camb.) 77:531–537.